Talanta xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

Talanta

journal homepage: www.elsevier.com/locate/talanta

Enzymatic amplification of oligonucleotides in paper substrates

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ARTICLE INFO

Keywords:
Isothermal amplification
Enzyme
Paper substrate
Oligonucleotides
Porosity
Quantum dots

ABSTRACT

Several solution-based methods have recently been adapted for use in paper substrates for enzymatic amplification to increase the number of copies of DNA sequences. There is limited information available about the impact of a paper matrix on DNA amplification by enzymatic processes, and about how to optimize conditions to maximize yields. The work reported herein provides insights about the impact of physicochemical properties of a paper matrix, using nuclease-assisted amplification by exonuclease III and nicking endonuclease Nt. Bbv, and a quantum dot (QD) - based Forster Resonance Energy Transfer (FRET) assay to monitor the extent of amplification. The influence of several properties of paper on amplification efficiency and kinetics were investigated, such as surface adsorption of reactants, and pore size. Additional factors that impact amplification processes such as target length and the packing density of oligonucleotide probes on the nanoparticle surfaces were also studied. The work provides guidance for development of more efficient enzymatic target-recycling DNA amplification methods in paper substrates.

1. Introduction

Paper-based analytical devices (PAD) have recently emerged as promising platforms for diagnostics in resource-limited settings, and nucleic acid-based bioassays are integral to some of these systems [1–3]. The attractiveness of paper as a substrate for bioassays includes: (1) availability of various porosities and pore sizes, thicknesses and wicking rates at low cost [4,5], (2) cellulosic paper is hydrophilic and has relatively weak non-specific binding [2], (3) patterning strategies, e.g. wax printing, are facile and low cost [4,6], (4) strategies for surface modification for biomolecule immobilization are well-established, and (5) fluidics in paper is based on capillary action and transport of solution may operate independently of external pumps.

Paper-based devices have attracted considerable attention for development of low-cost assays of nucleic acids as screening technologies at point-of-care (POC) settings [7–15]. A variety of paper devices with integrated readout systems including colorimetric [8,9], fluorescence [10,11]. electrochemical [10,11] and chemiluminescence [13] have been proposed for the detection of nucleic acids. However, the detection limit achieved by these devices (pM-nM) [14] are well above the levels encountered in biological samples (aM-fM) [16]. Therefore, an off-chip amplification step is usually required to increase the number of target molecules to a detectable level prior to implementation of the assay [14,15]. Such an amplification step complicates a protocol and renders the bioassay less attractive for POC diagnostics. Several reports have recently attempted to address the issue by integrating various nucleic acid amplification techniques into paper substrates [17–23]. These amplification methods, conducted in paper substrates with different properties, proved to be highly efficient with limit of detection achieving a single copy of target nucleic acid [24].

In contrast, several studies have reported contradictory results, indicating inhibition of enzymatic amplification on different paper matrices [20,25,26]. For instance, Rohrman et al. reported that the product yield of recombinase polymerase amplification (RPA) was reduced when done in a Whatman chromatography paper (CHR) in comparison with the amplification done in bulk solution, while amplification using a glass fiber (GF) substrate produced a quantity of product comparable to that obtained from bulk solution reactions [25]. The authors hypothesized that this disparity was due to the larger pore sizes of GF substrates. Linnes et al. investigated different DNA amplification methods using different substrates that included CHR, GF, nitrocellulose (NC) and polyethersulfone (PES). They reported that polymerase chain reaction (PCR) was completely inhibited in all substrates, while loop-mediated isothermal amplification (LAMP) and thermophilic helicase-dependent amplification (tHDA) was most successful in PES substrates [26]. The authors suggested that the PES substrate has the least surface binding affinity for nucleic acids and enzymes [26]. In contrast, a recent study by Liu et al. reported a 3-fold enhancement of rolling circle amplification (RCA) using NC membranes in comparison with the solution phase reaction [17]. This RCA experiment involved DNA hybridization to a surface-immobilized oligonucleotide, and the enhancement was attributed to the higher localized

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https://doi.org/10.1016/j.talanta.2018.02.107
Received 24 December 2017; Received in revised form 26 February 2018; Accepted 26 February 2018

concentration of the immobilized DNA strands [17]. An overview of similar reports about amplification in paper substrates indicates a paucity of information about the impact of chemical and physical properties of the paper matrix on the efficiency of enzymatic DNA amplification reactions.

In this report, the work is intended to provide direction for optimal design of oligonucleotide amplification by enzymatic target-recycling processes on/in paper substrates by determining the potential for impact of different factors that can influence the reactions within the matrix. It is clear that the extent of impact for different types of enzymes may vary between different paper matrices. This study offers some insights about general physicochemical properties of paper substrates, such as the significance of: adsorption of enzymes/nucleic acids; paper pore sizes; and spatial localization of reaction. The spatial location will be referred to as the reaction "phase", with the "solution-phase" being reaction in the pores of the paper matrix, and "surface-phase" being reactions using reagents that were deliberately immobilized onto the physical surfaces of the fibers of the paper matrix. The experimental work considers the effectiveness of enzymatic target recycling using exonuclease III and nicking endonuclease Nt. Bbv, which have recently garnered attention as amplification methods that may be suitable for POC settings [27]. Although, the use of these nucleases for DNA amplification in paper substrates has not been reported, their simple reaction schemes facilitate the interpretation of the results. A Forster Resonance Energy Transfer (FRET) – based assay using semiconductor quantum dots (QDs) as the donor served to monitor the extent of product production by the amplification process. This fluorescence detection strategy was previously demonstrated to provide for sensitive and specific biorecognition in paper substrates [28,29].

2. Materials and methods

2.1. Materials

All oligonucleotides were provided by Integrated DNA Technologies (Coralville, IA, USA), and are identified in Table 1. Exonuclease III (EXO), nicking endonuclease Nt. BbvCI (Bbv) and 10 × CutSmart buffer were from New England Biolabs (Ipswich, MA, USA) and used without further purification. Tide Quencher™ 3-maleimide was from AAT Bioquest, (Sunnyvale, CA, USA). Diethylaminoethyl (DEAE)-functionalized magnetic beads (MB, 1 μm) were from Bioclone Inc. (San Diego, CA). Green-emitting CdSe/ZnS core/shell quantum dots (PL at 518 nm) were from Cytodiagnostics (Burlington, ON, Canada). Hexahistidine-maleimide peptide sequences were from Canpeptide Inc. (Montreal, QC, Canada). Illustra NAP-5 size exclusion chromatography columns were from GE Life Sciences (Quebec, Canada). Amicon Ultra-0.5 centrifugal filters were from Fisher Scientific (Ontario, Canada). Whatman® cellulose chromatography papers (Grade 1, CHR-1, 200 × 200 mm), Whatman® cellulose filter papers grade 1 (Circular, 150 mm diameter), grade 3 (Circular, 55 mm diameter) and grade 5 (Circular, 55 mm diameter), Whatman® glass microfiber Grade A (GF/A, Circular 24 mm diameter), sodium tetraborate, L-glutathione (GSH, reduced, ≥ 98%), DTT, tetramethylammonium hydroxide solution (TMAH, 25% w/w in methanol), sodium (meta)periodate (NaIO4, ≥ 99%), sodium cyanoborohydride ($NaCNBH_3$, 95%), 1-(3-aminopropyl)imidazole (API, 98%), 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES, ≥ 99.5%), albumin from bovine serum (BSA, ≥ 98%) and Salmon Sperm DNA were from Sigma Aldrich (Oakville, ON, Canada). All buffer solutions were prepared using a water purification system (Milli-Q, 18 MΩ cm^{-1}), and were autoclaved prior to use. The buffer solutions included 100 mM tris-borate buffer (TB, pH 7.4), 50 mM borate buffer (BB, pH 7.4), and phosphate buffer (PB, pH 7.4).

Table 1
The oligonucleotide sequences.

Name	Sequence
MB	5′- /SH/-CTGAGCACAGTCCTCAGCGAAA -/Cy3/ – 3′
TGT – 1	5′- $(T)_5$ TTTCGCTGAGGACTGTGCT $(T)_5$ – 3′
TGT – 2	5′-$(T)_6$ AGCAGCTGAGGACTGTGCTCAG $(T)_2$ – 3′
TGT – 3	5′- $(T)_{21}$ TTTCGCTGAGGACTGTGCT $(T)_{20}$ – 3′
TGT – 4	5′- $(T)_{36}$ TTTCGCTGAGGACTGTGCT $(T)_{35}$ – 3′

(TGT – target).

2.2. Preparation of molecular beacon probes (MB)

Two molecular beacon probes were used in this study. A 22-mer oligonucleotide that was modified with Cy3 dye at the 3′-end and a thiol group at the 5′-end, was used to prepare the MB probes. The thiol group was first reduced via 500 × DTT in 1 × PBS (10 mM phosphate, 137 mM NaCl, 2.7 mM KCl, pH 7.4) for 2 h. The unreacted DTT was then removed by ethyl acetate extraction (4 times). To prepare MB-TQ conjugates, 20 equivalents of Tide Quencher 3-maleimide was added to MB oligonucleotides in PBS and the solution was shaken overnight. To prepare MB-QD probes, the MB oligonucleotide was first functionalized with a hexahistidine tag (H6) by incubating it with 5 M equivalents of a maleimide functionalized peptide (Maleimide-G(Aib)GHHHHHH, for 24 h. Unreacted TQ-3 and peptide was removed by running the sample through two consecutive NAP-5 desalting columns.

Immobilization of H6-oligonucleotides was performed using a solid-phase immobilization method that we have recently developed [30]. To obtain different packing densities of H6-MB on QD surfaces, various equivalents of oligonucleotides to QDs (3–40 eq) were added to the positively charged magnetic beads that were dispersed in TBS buffer (tris-borate 100 mM, NaCl 20 mM) at pH 7.4. To remove the unreacted oligonucleotides, the concentration of NaCl was increased to 350 mM. This process removed > 95% of the unreacted oligonucleotides (See Fig. S1). The DNA/QD values were determined by independent quantification of QDs and MBs, as previously described [30].

2.3. Modification of the paper substrates

The papers were prepared using a method previously developed in our group [8,11]. Paper substrates were patterned with wax using a Xerox ColorQube 8570DN solid ink printer. Each rectangular paper sheet was 60 mm by 26 mm in dimension and was printed to contain an array of 8 by 4 of circular reaction zones of 3 mm diameter (See Fig. S2). Printing on smaller circular filter papers was achieved after adhering the papers to regular printing paper using double-sided tape. The wax printed papers were subsequently heated in an oven at 120 °C for 2.5 min. Nitrocellulose substrates were prepared by nitrating the CHR-1 paper. The papers were soaked in a solution (1:1 by volume) of concentrated sulfuric acid (98%) and nitric acid (73%) for 30 min, and then neutralized using sodium bicarbonate and rinsed extensively with deionized water.

For solution-phase amplifications, the reaction zones on the paper were used without further chemical modifications. In contrast, the surface-phase amplification made use of functionalized paper. The reaction zones were modified to conjugate imidazole groups to the paper for immobilization of QD-MB probes. Imidazole surface modification was conducted in two steps. First, the cellulose was modified to contain aldehyde groups by two consecutive additions of 5 μL of aqueous solutions of $NaIO_4$ (50 mM) and LiCl (700 mM), with heating of the paper at 50 °C for 30 min, followed by rinsing with deionized water and drying at 50 °C in an oven. Next, the papers were functionalized with imidazole groups by spotting 5 μL of a solution containing API at 200 mM and $NaCNBH_3$ at 300 mM, in HEPES buffer pH 8. The reactions were allowed to proceed at room temperature for 1 h. The papers were then rinsed with borate buffer 50 mM pH 9.2 and stored in a desiccator for later use.